POSSIBLE EFFECTS OF METALS ON THE GROWTH OF *GRACILARIA* SP. (RHODOPHYTA)

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ABSTRACT

The effects of copper, zinc, and iron on the growth of *Gracilaria* sp. were studied *in vitro*. The *Gracilaria* sp. tissues were cultured under five different concentrations (0μM, 0.1μM, 0.5μM, 10.0μM and 50.0μM) of these metals. The growth rate and chlorophyll *a* concentration of the algae were measured weekly during a 21 days cultivation. Results showed that, there were significant difference between different concentrations of various trace metal towards alga mean growth rate and mean chlorophyll *a* concentration. The sensitivity of the trace metal was generally lower at low concentrations of the various trace metal. A reduction in the mean growth rate of *Gracilaria* sp. was found for high concentration (50.0μM) of these trace metals. In conclusion, this is a simple, sensitivity and reproducible test method for assessing the toxicity of copper, zinc and iron in brackish and marine environments.

Key words: *Gracilaria*, mean growth rate, mean chlorophyll *a* concentration, copper, zinc, iron.

ABSTRAK

Kesan kuprum, zink, dan ferum ke atas tumbesaran Gracilaria *sp. telah dikaji secara* in vitro. *Tisu Gracilaria sp.telah dikulturkan di dalam media mengandungi kuprum, zink, dan ferum dalam kepekatan 0μM, 0.1μM, 0.5μM, 10.0μM and 50.0μM. Kadar tumbesaran dan kepekatan klorofil* a *alga diukur setiap minggu dalam ujikaji selama 21 hari. Hasil kajian mendapati terdapat perbezaan yang signifikan di antara pelbagai kepekatan kuprum, zink dan ferum terhadap purata kadar tumhesaran dan purata klorofil* a Gracilaria. *Kesan kuprum, zink dan ferum ke atas alga adalah kecil pada kepekatan yang rendah. Kepekatan yang tinggi (50.0μM) boleh menyebabkan tumbesaran* Gracilaria *terbantut. Kesimpulannya, kajian ini adalah ringkas, dan boleh diulangi untuk mengkaji kesan keracunan kuprum, zink dan ferum di dalam keadaan air payau dan air laut.*

Kata kunci: Gracilaria, *purata kadar tumbesaran, purata kepekatan klorofil* a, *kuprum, zink, ferum.*

1.0 INTRODUCTION

Gracilaria is from the division of Rhodophyta (red algae), and the subclass Florideophycidae, as it is able to produce floridean starch as food reserve (Bold and Wynne, 1985). *Gracilaria* falls in the order Bangiales and family Bangiaceae.

The general characteristic of floridean reds is that they are all branching filaments (Sze, 1997). Floridean red algae have complex life cycles, and most have three life phases called alternation of generation which are the gametophyte, the carposporohyte, and the tetrasporophyte (Sze, 1997).

Red algae are often used as foods and as sources of phycocolloids (hydrocolloids) in a wide range of commercial products (Sze, 1997). *Gracilaria* is a popular food source among local people in Sarawak. Two important phycocolloids (or gums) derived from red algae are carrageenan and agar, which are both polymers of galactose. The principle source of agar are *Gelidium*, *Gracilaria*, *Pterocladia* and *Ahnfeltia*, which are harvested from natural populations, primarily in Asia and Latin America, and processed for commercial use in Asia (Sze, 1997). Agar are widely use as food gel, in packing canned foods, in pharmaceutical capsules, in the treatment of constipation, and as a medium for culturing microorganisms.

Besides commercial uses, red algae are important members of many benthic communities, from tropical to polar oceans (Sze, 1997). They are highly important as

primary producers of organic matter in aquatic environments because of their photosynthetic activities, and primary source of food and energy for aquatic organisms.

The availability of particular inorganic nutrients in sea water system often determines the level of algal growth. A shortage of a nutrient will result in symptoms of deficiency, and at very low supply, leads to early mortality. An access will cause injury, and at high levels, may cause toxicity and even death. Many of these metals can influence directly on various physiological and biochemical process (Cid *et al.*, 1994). Studies on heavy metals polluted waters have revealed that metal pollution decreases algal diversity, and alters algal species composition (Pawlik-Skowrońska, 2001).

There are few inorganic nutrients in the environment that are essential to all plants. According to Sze (1997), macroelements that required by all algae in relatively large quantities are carbon, hydrogen, oxygen, sulfur, potassium, calcium, magnesium, phosphorus, and nitrogen. They also need microelements in much lower quantities, often as cofactors in enzyme systems which include iron, manganese, copper, zinc, and molybdenum. However, most elements are available in excess amounts due to anthropogenic activities.

Between 1959 and 2000, a total of 82 articles have been published in toxicity studies related to substances and macroalgae (Eklund and Kautsky, 2003). However only 8% of the tested compounds were metal (Eklund and Kautsky, 2003). Haglund *et al.* (1996) recorded that plants are relatively poorly represented as test organisms, especially the macroscopic algae despite their being much exposed to discharges (especially industrial

effluents which contains much toxicant). There are also no standardized or commonly used biotest method available involving macroscopic algae.

The basis of using *Gracilaria* as a test organism lies in the previous physiological research. Besides, *Gracilaria* is abundant and has a long life. It is also easy to collect, able to accumulate pollutants and provide sufficient tissue for contaminant analysis (Lin and Liao, 1999). An important property of *Gracilaria* is the wide salinity range within which it grows with high growth rates (Haglund *et al.*, 1996). This enables the use of this method in both marine and brackish waters. Therefore, *Gracilaria* well suited as a potential test organism.

In this study, macroalga *Gracilaria* was chosen because of its high laboratory growth rates and its epiphyte resistance (Haglund *et al.*, 1996). The aim of the present study was to examine effects of different concentrations of trace metals (copper, zinc and iron) on the growth rate and chlorophyll *a* concentration, in-vitro.

2.0 MATERIALS AND METHODS

2.1 Establishment of *Gracilaria* stock culture

Wild thallus of *Gracilaria* was obtained from Siakap (*Latos calcarifer*) cage culture of Inland Fisheries Centre in Samariang Batu. The thallus was cultivated in seawater enriched with f/2 medium according to Appendix A.

Seawater used for the study was taken from the same cage culture site. The seawater was filtered with membrane filter 0.45µm, and dilluted with distilled water to approximately 23-26 psu, as measured with a refractometer. To each liter of filtered seawater, the following was added:

i.	$NaNO_3$	75.0g/L dH_2O	1.0mL
ii.	$NaH_2PO_4.H_2O$	5.0g/L dH_2O	1.0mL
iv.	f/2 Trace Metal Solution		1.0mL
v.	f/2 Vitamin Solution		0.5mL

The f/2 medium was changed every week. The culture was maintained at approximately 25°C and light (~12µ$Em^{-2}S^{-1}$) was provided from the top with fluorescent tubes. The culture was kept under 12 hours light: dark cycle.

2.2 Preparation of inoculums

One week before the start of the experiments, *Gracilaria* thallus from the stock culture (section 2.1) was transferred to seawater medium with the salinity of which the test would be carried out. During this period, the pH was maintained at 7.4.

2.3 Preparation of test solutions and test setup

Filtered and autoclaved seawater was diluted with distilled water to the desired salinity (23-26psu). Each liter of diluted seawater was supplemented with 1.0mL of $NaNO_3$ (75.0g/L dH_2O), 1.0mL of $NaH_2PO_4.H_2O$ (5.0g/L dH_2O), and 0.5mL of f/2 Vitamin Solution

The metals tested include copper, zinc, and iron. Copper was added as copper sulfide ($CuSO_4$), zinc as zinc sulfate ($ZnSO_4$), and iron as iron chloride ($FeCl_3$). The concentrations chosen for the test was, 0.1μM, 0.5μM, 10.0μM, and 50.0μM. Controls consisted of the f/2 culture medium with complete f/2 trace metal solution. Test solution with 0μM consisted of the culture medium without the test metal. The rest of the test solution was prepared according to the concentrations of the trace metals in each test (figure 1). The pH (7.4) and salinity (~ 23-26psu) of the test solution were checked after final additions.

Erlenmeyer glass flasks of 250mL volume were used and 200mL test solutions were added. Triplicates were made for each test concentration and the control.

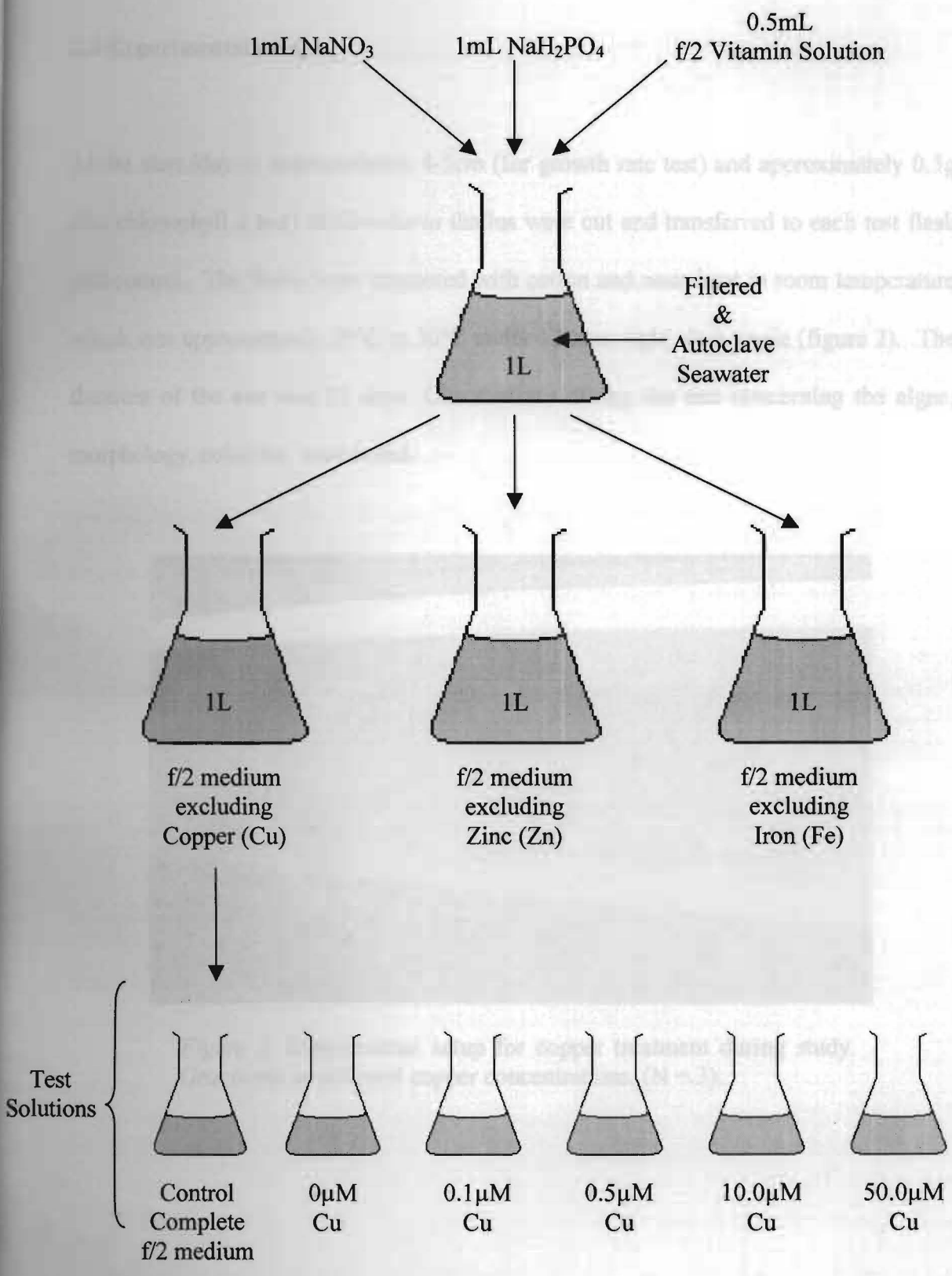

Figure 1: Preparation of test solutions.

2.4 Experimental design

At the start (day 0) approximately 4-5cm (for growth rate test) and approximately 0.5g (for chlorophyll *a* test) of *Gracilaria* thallus were cut and transferred to each test flask and control. The flasks were stoppered with cotton and were kept in room temperature which was approximately 26°C to 30°C under 12 hour light: dark cycle (figure 2). The duration of the test was 21 days. Observations during the test concerning the algae, morphology, color etc. were noted.

Figure 2: Experimental setup for copper treatment during study. *Gracilaria* in different copper concentrations. (N = 3).

2.4.1 Physiological responses measured

2.4.1.1 Growth rate

The fresh weight of the algae was determined at an interval of 7 days during the 21 days experiments. The algal specimens were carefully removed from the flasks with forceps and scrubbed clean of epiphytes and the excess water was blotted off with soft paper before weighing. This step was done quickly to avoid drying. Algae that were kept outside the flasks during the weighing procedure were stored in moist paper and returned as quickly as possible to the flasks.

The algal growth rate in each treatment was calculated from this formula:

$$\frac{W_t - W_0}{T_t - T_0} : \text{Growth Rate}$$

W_t = total fresh weight (g) of thallus at time t

W_0 = total fresh weight (g) of thallus at start

T_t = Time interval (days)

T_0 = Initial day

2.4.1.2 Chlorophyll *a* concentration

For comparison, an alternative method to the growth inhibition was examined. The method was based on chlorophyll *a* concentration measurements with spectrophotometer.

The chlorophyll *a* concentration of the algae was determined at an interval of 7 days during the 21 days experiments. The algal specimens were carefully removed from the flasks with forceps and scrubbed clean of epiphytes and the excess water was blotted off with soft paper. Then, the algal tissues were place into a tissue grinder, covered with 10ml of 90% acetone. Algal were grinded until completely homogenized. The homogenate was then filled into a labeled centrifuge tube (covered with tin foil) and stored in the fridge for 24 hours. After 24 hours, the homogenates were centrifuged at 4000rpm for 30 minutes. Small volumes of the acetone extract were filled into a glass photometer cuvette. Cuvette was placed into a spectrophotometer and absorbency was determined at the wavelength of 750, 664 and 647. The concentration of chlorophyll *a* was calculated using the formula of Jeffrey and Humphrey (1975):

$$\text{Chlorophyll } a \text{ (mg/g)} = \frac{11.93(A664) - 1.93(A647)}{0.5}$$

2.5 Data analysis

The growth response and chlorophyll *a* concentration in different concentrations on a particular week were analyzed. Data were statistically analyzed by a one-way analysis of variance (ANOVA) and, when differences observed were significant, means were compared by the multiple range Turkey test, at a level of significance of 0.05, using the SPSS software.

3.0 RESULTS

3.1 The growth of stock cultures in seawater enriched with f/2 medium.

Stock of *Gracilaria* cultured in seawater enriched with f/2 medium showed changes in morphology and color. The color of the algae changed from dark to lighter brown. This may be due to the light intensity and the salinity of the water. The salinity of the seawater used was approximately 23 to 26 psu, which was lower than the ambient salinity where it was obtained (approximately 27 to 30 psu).

3.2 Response of the *Gracilaria* in different concentrations of copper, zinc and iron.

3.2.1 Morphology

It was observed that the algae kept in the control medium containing 0.039μM copper showed a slow and steady growth. Algae kept in 0μM, 10.0μM and 50.0μM of copper shown a little pale end at the thallus (figure 3). The thallus kept in 0.1μM and 0.5μM was brown in color and was in good condition. Towards the end of 21 days, the morphology of algal thallus in zinc test medium deteriorated. The thallus showed signs of loosing pigmentations. Morphology of algal thallus kept in different concentrations of iron had been overall in good conditions. The thallus was brown in color and looked healthy (figure 4).

Overall, epiphytes had been a problem through out this experiment. Changes in morphology were seen in some of the cultures which were attacked by epiphytes. When looked under a microscope, the epiphytes were seen to be like diatom (figure 5), and small filaments of vegetation (figure 6) which were not identified. However, these epiphytes were removed as much as possible at every 7 days before readings of the growth rates were taken. Unfortunately still the epiphytes did cause some damage to the thallus such as loss of pigmentation, which might interfere with the readings.

Figure 3: Arrows showed algal thalli loosing its pigments when cultured in high concentrations (50.0μM) of copper solution.

Figure 4: Healthy algal thallus in 0.5μM iron solution.

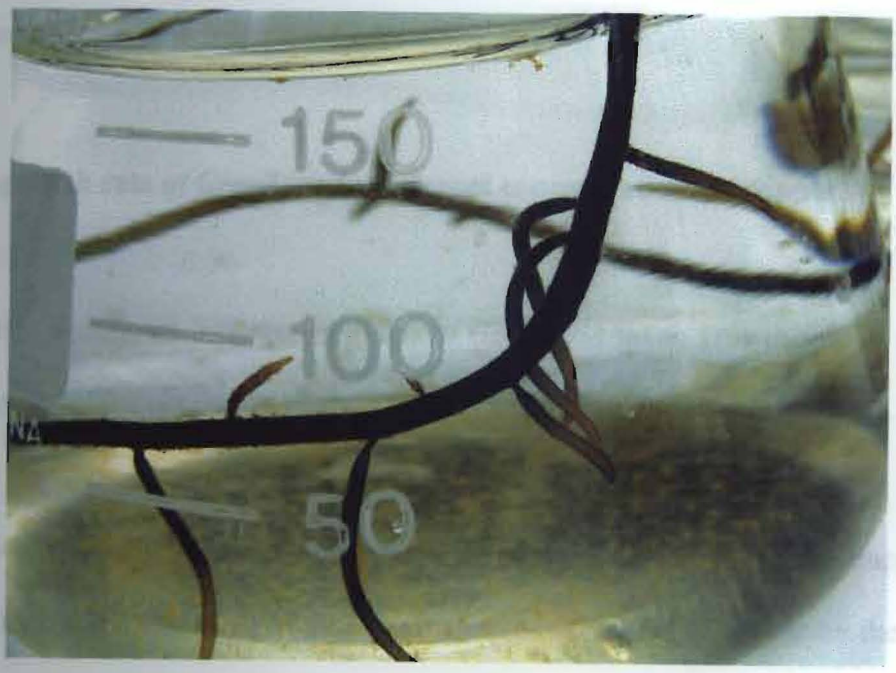

Figure 5: Diatoms seen on the *Gracilaria* and at the bottom of the flask.

Figure 6: Arrows showed filamentous algae growing on the thallus of *Gracilaria.*

3.2.1 Growth Rate

a. Mean growth rate of *Gracilaria* in different concentrations of copper

There was a significant different ($p<0.05$) in *Gracilaria* mean growth rate in different concentrations of copper on the 7th day, 14th day and 21st day (Table 1).

Test showed that algae mean growth rate in medium containing 0μM copper was significantly different from algae mean growth rate in 50.0μM of copper on the 7th day, as its mean growth rate was the highest (0.303g) compared to algae in 50.0μM copper, which had the lowest mean growth rate of 0.005g as shown in figure 7.

On the 14th day, test showed that algae mean growth rate in medium containing 0μM copper was significantly different from algae in all other concentrations of copper as its mean growth rate was the highest (0.028g). Where as algae in medium containing 50μM copper did not show any increase in its mean growth rate.

On the 21st day, algae mean growth rate in 0.1μM and 0.5μM of copper were significantly different from that of control, 0μM, 10.0μM and 50.0μM of copper. Algae in 0.1μM copper has the highest mean growth rate (0.022g) followed by algae in 0.5μM copper test solution (0.015g). However no growth was observed for algae in 10.0μM and 50.0μM copper.

b. Mean growth rate of *Gracilaria* in different concentrations of zinc

There was a significant different ($p<0.05$) in *Gracilaria* mean growth rate in different concentrations of zinc on the 7th day, and 21st day. However, in the second week which was on the 14th day, there was no significant different in *Gracilaria* mean growth rate (Table 2).

On the 7th day, algae in 50.0μM zinc were significantly different from algae in all other zinc concentrations. Its mean growth rate was the highest (0.007g), where as algae in control (0.077μM), 0μM, and 0.1μM of zinc showed small increase in their mean growth rate, 0.002g, 0.001g and 0.001g respectively (figure 8). No growth was yet to be observed for algae in 10.0μM and 50.0μM zinc test solution.

On the 14th day, there was no significant different between the mean growth rate of algae in control and test mediums. Algae in all the different test medium had shown an increase in mean growth rate. However, algae mean growth rate in 50.0μM zinc had decreased from 0.003g on the 14th day compared to 0.007g on the 7th day.

On the 21st day, mean growth rate of algae in control, 0μM, 0.1μM and 0.5μM zinc were significantly different from mean growth rate of algae in 10.0μM and 50.0μM zinc. Overall, mean growth rate for algae in control, 0μM, 0.1μM and 0.5μM zinc had increased compared to that on the 14th day. However algae in 10.0μM and 50.0μM zinc had shown a decrease in mean growth rate compared to that on the 14th day. Algae in

control shown the highest growth rate (0.018g) followed by algae in 0μM zinc (0.015g) and 0.5μM zinc (0.013g). No growth was observed for algae in 50μM zinc test solution.

c. Mean growth rate of *Gracilaria* in different concentrations of iron

Overall, there was a significant different ($p<0.05$) in *Gracilaria* mean growth rate on the 7th day and on the 14th day. However, on the 21st day, *Gracilaria* mean growth rate was not significantly different between the different concentrations of iron (Table 3).

On the 7th day, *Gracilaria* in 0μM and 0.1μM iron had the highest mean growth rate of 0.006g respectively. No growth was yet observed for algae in 0.5μM, 10.0μM and 50.0μM of iron test solutions.

On the 14th day, *Gracilaria* mean growth rate in 0.5μM and 10.0μM iron was significantly different from that in control (12.0μM) and other concentrations of zinc test solutions. *Gracilaria* in 0.5μM iron had the highest increase in mean growth rate (0.011g) followed by algae in 10.0μM iron (0.010g) (Figure 9).

On the 21st day, algae in test medium showed an increase in mean growth rate. Algae in 10μM iron had the same mean growth rates as that in control (0.011g).

Table 1. A summary of the one-way ANOVA for the weekly growth of *Gracilaria* sp. under different levels of copper concentrations.

Between Groups	Mean Square	F	P
Day 7	0.000	3.884	0.025
Day 14	0.000	13.407	0.000
Day 21	0.000	26.989	0.000

Table 2. A summary of the one-way ANOVA for the weekly growth of *Gracilaria* sp. under different levels of zinc concentrations.

Between Groups	Mean Square	F	P
Day 7	0.000	16.680	0.000
Day 14	0.000	0.676	0.650
Day 21	0.000	10.388	0.000

Table 3. A summary of the one-way ANOVA for the weekly growth of *Gracilaria* sp. under different levels of iron concentrations.

Between Groups	Mean Square	F	P
Day 7	0.000	3.630	0.031
Day 14	0.000	5.856	0.006
Day 21	0.000	2.386	0.101

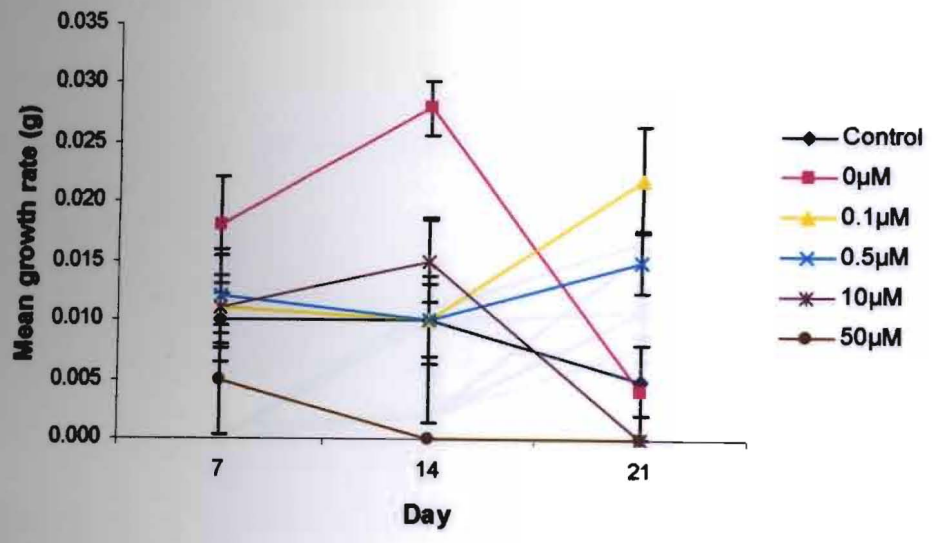

Figure 7: Mean growth rate of *Gracilaria* sp. on the 7th, 14th and 21st day in different concentrations of copper. Bars represent the standard deviation. Sample size, N = 3

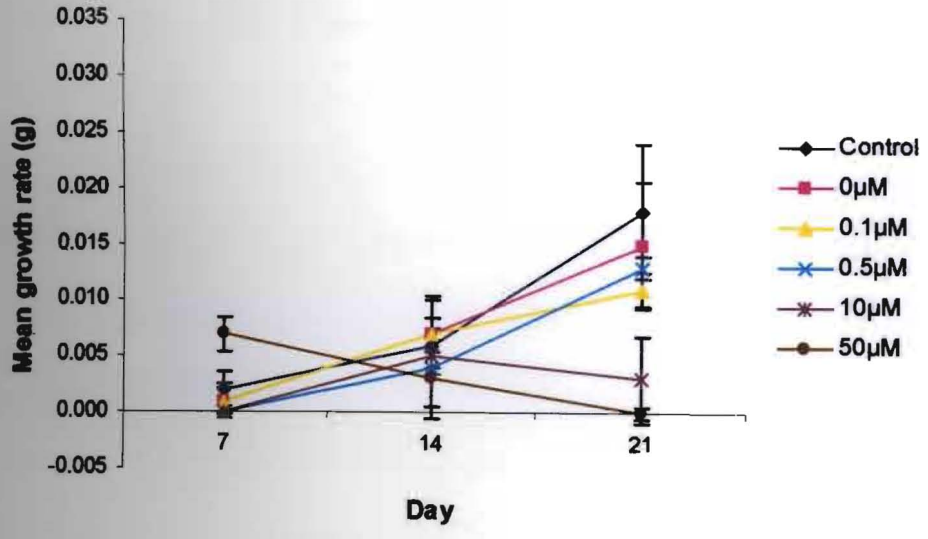

Figure 8: Mean growth rate of *Gracilaria* sp. on the 7th, 14th and 21st day in different concentrations of zinc. Bars represent the standard deviation. N = 3

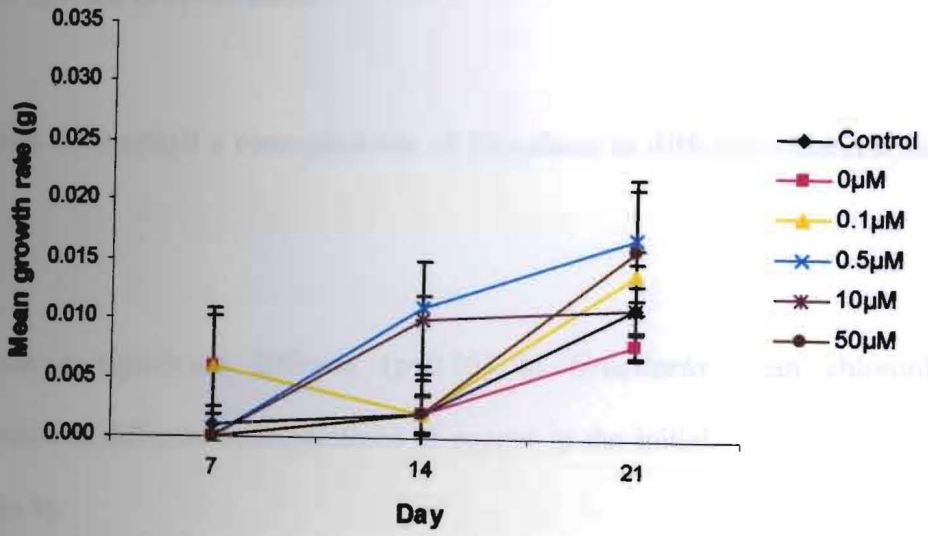

Figure 9: Mean growth rate of *Gracilaria* sp. on the 7th, 14th and 21st day in different concentrations of iron. Bars represent the standard deviation. N = 3

3.3 Chlorophyll *a* concentration

3.3.1 Mean chlorophyll *a* concentration of *Gracilaria* in different concentrations of copper.

There was a significant different ($p<0.05$) in *Gracilaria* mean chlorophyll *a* concentration in different concentrations of copper in the initial, 7th day, 14th and 21st day (Table 4).

On the 7th day, all algae showed an increase in the mean chlorophyll *a* concentration except that in 0μM copper as shown in the Figure 10. The mean chlorophyll *a* concentration in 0μM test medium decrease from 12.55mg/g in the initial day to 11.89 on the 7th day. However, the following observations showed the algae in 0μM copper showed increased in chlorophyll *a* concentration on the 7th day.

On the 14th day, algae in control showed a high increase in mean chlorophyll *a* concentration, from 11.13mg/g on the 7th day to 23.24mg/g on the 14th day. However, algae in 10.0μM and 50.0μM copper showed a decrease in the mean chlorophyll *a* concentration from 17.41mg/g to 16.22mg/g and 18.73mg/g to 16.16mg/g respectively.

On the 21st day, algae in control and all test solutions showed an increase in its mean chlorophyll *a* concentration. Overall, algae in 0.5μM copper showed a consistence increase in its mean chlorophyll *a* concentration.

3.3.2 Mean chlorophyll *a* concentration of *Gracilaria* in different concentrations of zinc.

There was a significant different in mean chlorophyll *a* concentration of *Gracilaria* growing in different zinc concentration used on the 7th day, 14th day and 21st day (Table 5).

On the 14th day, *Gracilaria* in 0.5μM, 10.0μM, and 50.0μM zinc showed very small increase in chlorophyll *a* concentration, 0.72mg/g, 0.20mg/g and 0.99mg/g respectively, in contrast to that in the control (0.077μM), which increased 9.0mg/g chlorophyll *a* concentration when compared to that on the 7th day (figure 11). *Gracilaria* in 0μM zinc showed small increase in mean chlorophyll *a* concentration (2.61mg/g).

On the 21st day, chlorophyll *a* concentration of *Gracilaria* increased. However, *Gracilaria* in 50.0μM zinc showed a reduction in its mean chlorophyll *a* concentration.